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The PI3K/Akt pathway is present and functional in the preimplantation mouse embryo

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Abstract

The PI3K/Akt signal transduction pathway is a well-known mediator of growth promoting and cell survival signals. While the expression and function of this pathway have been documented during early and late stages of the reproductive process, currently, there is no evidence demonstrating either the presence or function of the PI3K/Akt pathway in murine preimplantation embryos. We found, using confocal immunofluorescent microscopy and Western blot analysis, that the p85 and p110 subunits of PI3K and Akt are expressed from the 1-cell through the blastocyst stage of murine preimplantation embryo development. These proteins were localized predominantly at the cell surface from the 1-cell through the morula stage. At a blastocyst stage, both PI3K and Akt exhibited an apical staining pattern in the trophectoderm cells. Interestingly, phosphorylated Akt was detected throughout murine preimplantation development, and its presence at the plasma membrane is a reflection of its activation status. Inhibition of Akt activity had significant effects on the normal physiology of the blastocyst. Specifically, inhibition of this pathway resulted in a reduction in insulin-stimulated glucose uptake. In addition, inhibiting Akt activity resulted in a significant delay in blastocyst hatching, a developmental step facilitating implantation. Finally, we established the presence of this pathway in trophoblast stem (TS) cells, a potentially useful in vitro model to study this signaling cascade. Taken together, these data are the first to demonstrate the presence and function of the PI3K/Akt pathway in mammalian preimplantation embryos.

Keywords: PI3K/Akt; Preimplantation mouse embryo; Trophoblast stem; Blastocyst hatching

Introduction

The mammalian preimplantation embryo is a critical and unique stage in embryonic development. It is at this time that a number of significant developmental milestones are achieved, including the first mitotic divisions, the establishment of cellular contacts and the differentiation of the first cell lineages. The unique environment in which the preimplantation embryo develops further characterizes this stage. At this time, the embryo is free-floating, having no direct cell to cell contact, lacks a blood supply and is exposed to a dynamic fluid environment (Hardy and Spanos, 2002). The embryo is dependent on autocrine and paracrine growth factors to support its growth and development.

Four days post-fertilization (in the mouse), the embryo has reached the blastocyst stage of development, the point in embryonic development when the first cell differentiation step has occurred. At this stage, the embryo is comprised of the epithelial trophectoderm (TE), which is a layer of cells that surrounds the embryo and will develop into the extra embryonic tissue, and the inner cell mass (ICM), which consists of the pluripotent cells that will later differentiate

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into the fetus. It is vital for continued embryonic development, and the maintenance of pregnancy that an effective maternal–embryonic interface is established.

Prior to implantation, the developing embryo is dependent on signals generated by growth factors present in the maternal environment. These growth factors are known to regulate cell proliferation and differentiation during mammalian preimplantation development (Raff, 1992; Weil et al., 1996). Embryos are able to respond to these growth factors because they express many of the cognate receptors for the ligands present in the maternal tract and those synthesized by the embryo itself (Dardik et al., 1992). Although embryos are capable of developing in vitro, their development is significantly delayed relative to development in vivo, suggesting roles for both embryo- and maternal-derived growth factors (Bowman and McLaren, 1970; Paria and Dey, 1990). This hypothesis is also supported by the fact that culturing embryos at high density or in the presence of specific growth factors can partially reduce the developmental delay (Adamson, 1993). For example, transforming growth factor-α (TGF-α), whose cognate receptor is expressed from a 4-cell stage on, has been shown to significantly enhance in vitro development (Dardik et al., 1992; Paria and Dey, 1990; Rappolee et al., 1988; Wiley et al., 1992).

During this preimplantation period, the embryo expresses a number of receptors known to activate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Specifically, the IGF-1 and insulin receptors are expressed from the 1-cell stage onward (Lighten et al., 1997; Smotrich et al., 1996). Over the past decade, the PI3K/Akt cascade has been extensively studied and implicated as a key regulator of a number of cellular functions, including cellular migration, mitogenesis, differentiation and cell survival (Franke et al., 1997). Initially implicated in the suppression of apoptosis in growth-factor-mediated survival of the pheochromocytoma cell line PC12, PI3K activity was subsequently found to be required for the growth-factor-dependent survival of a wide variety of cell lines ranging from fibroblasts to neurons (Crowder and Freeman, 1998; Scheid et al., 1995; Stambolic et al., 1998; Vemuri and McMorris, 1996; Yao and Cooper, 1995). Although a number of molecular substrates for PI3K have been identified, it is believed that Akt is the primary target responsible for mediating an antiapoptotic signal. Akt is a pivotal player in multiple cellular signaling pathways and acts as a transducer of many functions initiated by growth factor receptors that activate PI3K.

Akt, a serine threonine kinase, is the cellular homolog of the transforming oncogene of the AKT8 retrovirus (Staal, 1987). There are three known Akt family members (Akt1–3) that are differentially expressed in a variety of tissues (Bellacosa et al., 1993). Akt family members share a common structure that consists of an N-terminal regulatory pleckstrin homology (PH) domain, a hinge region connecting the PH domain to a kinase domain with serine–threonine specificity, and a C-terminal region required for the induction and maintenance of its kinase activity (Ahmed et al., 1993; Chan et al., 1999; Franke et al., 1994).

Although these family members are highly homologous, the phenotypes of Akt1 and Akt2 deficient mice are distinct, implying that these proteins have separate physiological roles. Akt1 knockout mice are growth-retarded and exhibit increased levels of apoptosis, but they do not have metabolic defects (Chen et al., 2001; Cho et al., 2001b). In contrast, Akt2 knockout mice are insulin resistant and display a diabetic phenotype (Cho et al., 2001a). A deficiency in both Akt1 and Akt2 results in perinatal lethality. These mice are extremely growth-retarded, they exhibit atrophy in multiple organ systems and display failed adipogenesis (Peng et al., 2003). Thus, even though Akt isoforms are activated similarly and phosphorylate downstream substrates with equal specificity and efficiency (Franke, 2000), the aforementioned data suggest that these kinases have distinct biological roles, namely, Akt1 is involved in the transduction of growth signals, while Akt2 is critical for glucose homeostasis.

Although PI3K and Akt expression have not been characterized in mammalian preimplantation embryos, the expression and function of these kinases have been explored in the reproductive system of animals at times other than preimplantation embryo development. Studies conducted in various animal models have demonstrated that PI3K and Akt play an important role in oocyte maturation. Previously, it was shown that PI3K and Akt are involved in FSH-induced meiotic maturation in mice (Hoshino et al., 2004). In addition, data generated in a porcine animal model suggested that PI3K activity in cumulus cells is required for both the suppression of spontaneous meiotic resumption and the induction of gonadotropin-stimulated meiotic resumption (Shimada et al., 2003). Finally, PI3K/Akt was shown to induce the resumption of meiosis in both *Xenopus* and starfish oocytes (Andersen et al., 1998; Hiraoka et al., 2004). Aside from its role in oocyte maturation, this signal transduction pathway also appears to be involved in the mechanism by which certain cytokines mediate their antiapoptotic effects. The antiapoptotic effect of stem cell factor on oocytes in primordial follicles was shown to involve the PI3K/Akt signal transduction pathway (Jin et al., 2005).

PI3K and Akt may also play a role later in the reproductive process. Akt1 is widely expressed in murine placenta including trophoblast and vascular endothelial cells. Akt1 deficient mice display defects in placental development including decreased vascularization, hypotrophy, an absence of glycogen-containing cells in the spongiotrophoblast and a reduction of the decidual basalis (Yang et al., 2003). Moreover, the PI3K/Akt pathway has been implicated in both the differentiation and migration of trophoblast cells (Kamei et al., 2002; Qiu et al., 2004). Thus, taken together, these data suggest the PI3K/Akt pathway

may be important at various stages during the reproductive process and therefore may be important for reproductive success.

The aforementioned studies characterizing the role of the PI3K/Akt pathway during reproduction lead us to examine whether these proteins are present and functional during murine preimplantation development. The identification of the presence of this pathway in preimplantation embryos may prove important for elucidating signal transduction pathways that are crucial for preimplantation embryo survival and reproductive success.

Materials and methods

Embryo recovery and culture

All mouse studies were approved by the Animal Studies Committee at Washington University School of Medicine and conform to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Embryos were recovered as previously described (Moley et al., 1998). Briefly, female mice (B6 X SJL F1, Jackson Laboratories, Bar Harbor, ME) 4–6 weeks of age were given free access to food and water and were maintained on a 12 h light/dark cycle. Female mice were superovulated with an intraperitoneal (ip) injection of 10 IU/animal of pregnant mare serum gonadotropin (PMSG) (Sigma Chemical Co, St. Louis, MO) followed 48 h later by an ip injection of 10 IU/animal of human chorionic gonadotropin (hCG) (Sigma Chemical Co.) and mated with males of proven fertility. Mating was confirmed by identification of a vaginal plug. Animals were sacrificed at 24 (one-cell), 48 (two-cell), 54 (four-cell), 72 (morula) or 96 (blastocyst) h post-hCG administration and mating to isolate embryos at the appropriate stage of development. Embryos were obtained by flushing dissected uterine horns and ostia and immediately placed in human tubal fluid medium (HTF) (Irvine Scientific, Irvine, CA) supplemented with 0.25% BSA (fraction V, Sigma; St. Louis, MO). One-cell embryos were immediately placed in HTF medium containing 0.3 mg/ml hyaluronidase (Sigma; St. Louis, MO) to remove the cumulus cells. Further treatment of embryos for specific experiments is described below.

Immunofluorescent staining of embryos

For localization of the PI3-kinase subunits (p85 and p110), Akt and phospho-Akt during preimplantation development, embryos were fixed for 20 min in 3% paraformaldehyde (Sigma; St. Louis, MO), permeabilized for 20 min with 0.1% Tween-20 (Sigma; St. Louis, MO) and placed on glass slides. All staining was performed on slides under microdroplets in a humidified chamber at room temperature in the dark. Fixed embryos were blocked with 20% normal goat serum or 20% normal donkey serum in phosphate-buffered saline (PBS) with 2% BSA for 1 h. Embryos were immunostained with goat anti-PI3K p110 (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA), rabbit anti-PI3K p85 (Upstate Cell Signaling Solutions; Lake Placid, NY), rabbit anti-Akt (Akt1/2) (H-136, Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) or rabbit anti-phospho-Akt1/2/3 (serine 473) (Covance Research Products, Inc.; Berkley, CA) at a concentration 20 μg/ml for 1 h. After three 10 min washes with PBS/2%BSA, an anti-rabbit or anti-goat Alexa 488 secondary antibody was added at a concentration of 2 μg/ml (Molecular Probes, Eugene, OR) for 20 min. Embryos were counterstained with TO-PRO®-3 iodide (Molecular Probes; Eugene, OR) at a concentration of 4 μM for 20 min. Finally, embryos were washed 3 times in PBS and mounted in drops of Vectashield (Vector Laboratories; Burlingame, CA) under a coverslip. Fluorescence was detected with laser-scanning confocal immunofluorescent microscopy (Bio-Rad MRC-600, 63× magnification) as described previously (Moley et al., 1998). As controls, fixed embryos were stained with control preimmune sera or affinity-purified whole IgG. All experiments were performed at least three times with representative experiments presented.

Embryo Western blot analysis

An average of 75 embryos were pooled, added directly to Laemmli sample buffer, subjected to SDS-PAGE and transferred to nitrocellulose. Blots were blocked for 1 h at RT in 5% milk in TBS-T. The blots were probed overnight at 4°C in 1% milk in TBS-T with the following antibodies as indicted in the figure legends: rabbit anti-PI3-kinase p85 (1 μg/ml) (Upstate, Lake Placid, NY); rabbit anti-p-Akt (Ser 473) 0.5 μg/ml (Covance Research Products, Inc.; Berkley, CA); and mouse anti-actin (1:1000) (Chemicon International Inc.; Temecula, CA). Blots were washed 3 times for 10 min each with TBS-T. The appropriate HRP-conjugated secondary antibody (either goat anti-rabbit or goat anti-mouse) at a dilution of 1:10,000 was used for detection (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using SuperSignal® West Dura following the manufacturer's instructions (Pierce; Rockford, IL).

To determine if Akt was activated in response to insulin stimulation, blastocysts were recovered as described above and then immediately incubated for 30 min in HTF/BSA or HTF/BSA supplemented with 1 μM insulin (Sigma; St. Louis, MO). Blastocysts were then rinsed in HTF only and stored at −80°C until Western blot analysis was performed. To demonstrate the effect of LY-294002 on the phosphorylation status of Akt, blastocysts were harvested as described above and cultured in either DMSO (vehicle control) or 250 μM LY-294002 for 15 or 30 min and subsequently subjected to Western blot analysis. These experiments were conducted at least three times each.

Insulin-stimulated glucose uptake in blastocysts

To examine the physiologic function of the PI3K/Akt pathway in the blastocyst, embryos were exposed to PI3K inhibitors, and IGF-1/insulin-stimulated glucose uptake was assessed. Nonradioactive insulin-stimulated 2-deoxyglucose uptake into single blastocysts was performed using microfluorometric assays combined with enzymatic cycling reactions as previously described (Moley et al., 1998). Briefly, embryos were cultured for 10 min in HTF only, HTF supplemented with 250 μM LY-294002 or HTF supplemented with 100 nM Wortmannin (Sigma; St. Louis, MO). Next, blastocysts from each condition were incubated in HTF media at a final glucose concentration of 5.6 mM with 500 nM insulin (Sigma, bovine pancreas) for 30 min. 2-Deoxyglucose uptake was then measured as described previously (Moley et al., 1998).

Blastocyst hatching

Blastocysts were harvested and subsequently cultured in vitro at 37°C under 5% CO_2 in plates containing 30 μl droplets of HTF medium under oil. A total of ten unhatched blastocysts were placed in each droplet. The droplets contained HTF plus DMSO (vehicle control) or 250 μM LY-294002. Blastocyst hatching was examined after 24 h in culture using phase-contrast microscopy. A minimum of 100 blastocysts was examined per treatment group over the course of three independent experiments.

Trophoblast stem (TS) cell culture

Primary TS cells were a generous gift from Dr. Leonidas Carayannopoulos (Washington University, St. Louis MO) and were generated from C57/B6 mice following published protocols (Tanaka et al., 1998). To confirm the trophoblast lineage of this cell line, several genetic markers were analyzed. These markers included two TS specific genes, *Errβ* and *Hand1*, which were both expressed in this cell line, one embryonic stem (ES) cell specific marker, Oct3/4, which was not expressed in this cell line, and one mesoderm specific gene, *Brachyury*, which was not expressed in this cell line. In addition, this TS line was able to differentiate to become *Placental lactogen 1* (PL-1) positive, a marker of giant cells. TS lines were maintained in the absence of mouse embryonic fibroblast (MEF) feeder layers, cultured instead in the presence of MEF conditioned media (MEF-CM). MEF-CM was generated by culturing 2×10^6 irradiated MEFs (5000 rads) per 10 cm dish in TS media [RPMI (Cambrex Bio Science; Walkersville, MD), 20% FBS (Hyclone; Logan, UT), 1 mM sodium pyruvate (Cambrex Bio Science; Walkersville, MD), 100 μM β-mercaptoethanol (Sigma; St. Louis, MO) and 2 mM L-glutamine (Cambrex Bio Science; Walkersville, MD)] for 72 h. Medium was spun down to remove floating cells and debris, filtered (0.2 μm) and frozen in 50 ml aliquots at −20°C. TS cells were then cultured in 70% MEF-CM and 30% TS medium supplemented with 25 ng/ml fibroblast growth factor-4 (FGF-4) (Sigma; St. Louis, MO) and 1 μg/ml heparin (Sigma; St. Louis, MO).

To identify the presence of the PI3K p85 subunit, Akt and p-Akt in the TS cells, 2×10^6 cells were cultured as described above and lysed in a buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA and protease inhibitors. 10 μg of protein was loaded per lane as determined by the BCA protein assay (Pierce; Rockford, IL) and subsequently subjected to Western blot analysis. All antibody concentrations and Western blot conditions were the same as described for Western blot analysis of embryos. To demonstrate the effect of LY-294002 on the phosphorylation status of Akt, 2×10^6 TS cells were cultured in either DMSO or 250 μM LY-294002 for 15, 30 or 60 min and subsequently subjected to Western blot analysis.

Statistical analysis

For evaluation of differences in IGF-1/insulin-stimulated 2-deoxyglucose uptake, ANOVA was utilized with a Fisher post-hoc test. An unpaired *t* test was used for the analysis of the in vitro hatching experiments. Results are expressed as means ± standard error of at least three separate experiments.

Results

PI3-kinase and Akt expression in preimplantation development

To date, there are no data showing the expression of phosphatidylinositol 3-kinase (PI3K) in the mammalian preimplantation embryo. In order to elucidate the presence of this kinase at this early stage of development, we first performed immunofluorescent staining on preimplantation embryos from a one-cell to a blastocyst stage (Fig. 1). PI3K is a heterodimeric enzyme composed of an 85 kDa regulatory subunit and an 110 kDa catalytic subunit. Fig. 1 illustrates the expression of both subunits of this kinase at all stages of preimplantation development. Both subunits are predominantly localized to the plasma membrane (PM) of cleavage stage embryos. During this period, these proteins were also detected in the cytoplasm of the developing embryo. At the blastocyst stage, the p85 and p110 subunits are largely expressed on the apical surface of the trophectoderm (TE). Thus, both PI3K subunits are expressed throughout murine preimplantation development.

The best characterized downstream target of PI3K is the serine–threonine kinase, Akt, a well-known mediator of survival signals. Similar to PI3K, its expression in the mammalian preimplantation embryo has not been charac-

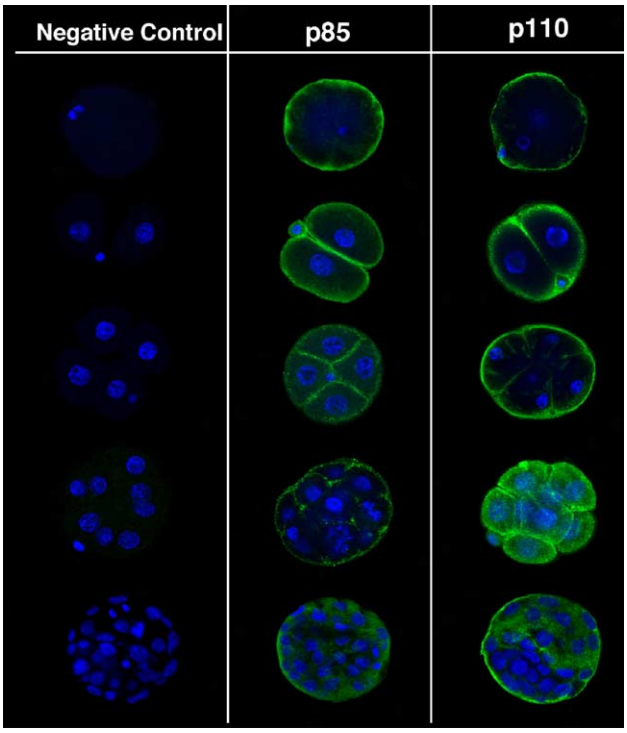

Fig. 1. Detection of PI3K subunits, p85 and p110, in preimplantation embryos. Preimplantation mouse embryos were retrieved at different stages of development and stained with either preimmune antisera as a negative control or with antibodies specific for the p85 or p110 subunit of PI3K. The embryos were then incubated with a secondary antibody, Alexa Fluor 488 goat anti-rabbit or donkey anti-goat IgG (green fluorescence). Embryos were counterstained with TO-PRO-3 iodide, which stains nuclei (blue channel).

terized. Immunofluorescent staining of preimplantation embryos using an antibody specific for Akt1 and Akt2 was utilized to determine the presence and expression pattern of this protein. As seen for PI3K, Akt is expressed at all stages of development and appears to be expressed predominantly at the PM (Fig. 2). Again, at the blastocyst stage, Akt displays an apical pattern of expression on the TE. As shown in Fig. 2, phosphorylated Akt is detected throughout murine preimplantation development, and its presence at the plasma membrane is a reflection of its activation status. Protein expression of the PI3K subunits, Akt, and the phosphorylation status of Akt were confirmed by Western blot analysis of blastocyst stage embryos (Fig. 3).

Akt is activated in response to insulin

In order to determine if preimplantation embryos respond to growth factor stimulation by activating Akt, blastocysts were cultured in the presence of insulin and analyzed for an increase in Akt phosphorylation as compared to untreated control embryos. As depicted in Fig. 4, there is an average 2-fold increase in the amount of phosphorylated Akt in embryos stimulated with insulin. This confirms that the PI3K/Akt pathway is active at this early stage of development. To further characterize this pathway in preimplanta-

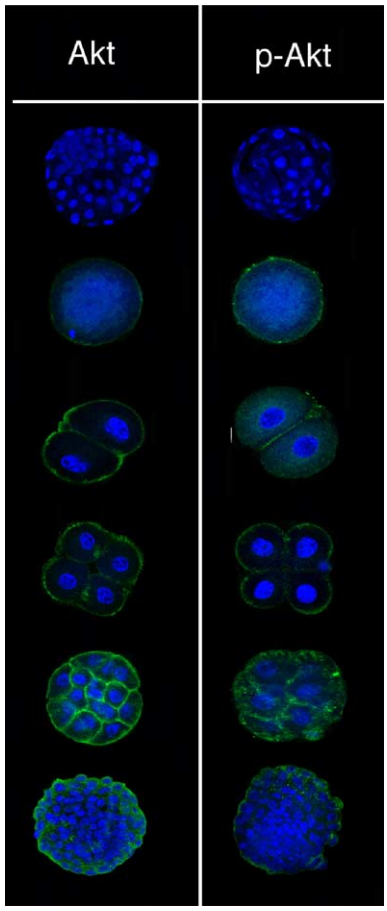

Fig. 2. Immunofluorescent staining of Akt and phospho-Akt. Preimplantation mouse embryos were retrieved at different stages of development and stained with antibodies specific for Akt (Akt1/2) or p-Akt (Ser 473). The embryos were then incubated with a secondary antibody, Alexa Fluor 488 goat anti-rabbit (green channel). Embryos were counterstained with TO-PRO-3 iodide, which stains nuclei (blue channel).

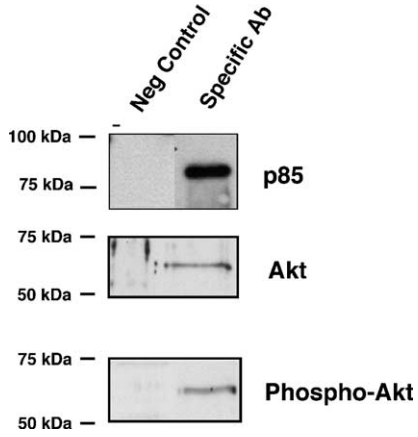

Fig. 3. Detection of the PI3K p85 subunit, Akt and p-Akt in preimplantation blastocyst by Western blot analysis. An average of 75 blastocyst stage embryos/well were added to Laemmli sample buffer and then subjected to SDS-PAGE and Western blot analysis. The membranes were probed with preimmune sera or antibodies specific for p85, Akt (Akt 1/2) or p-Akt.

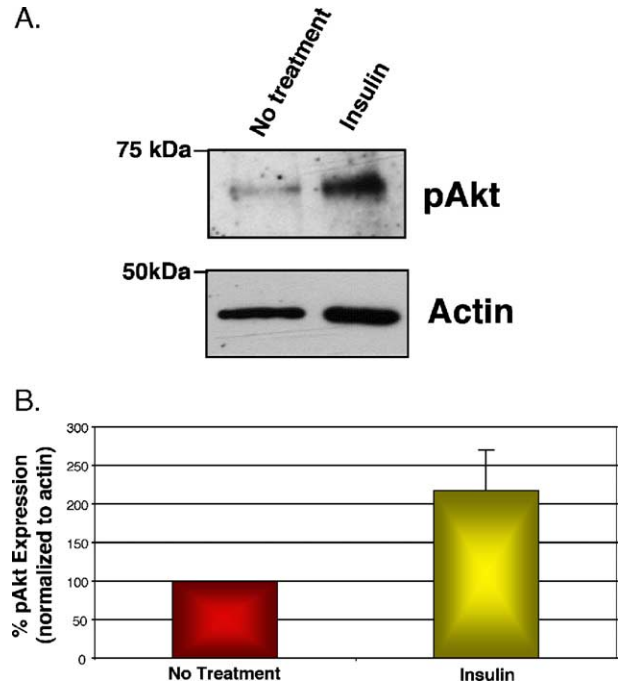

Fig. 4. Preimplantation embryos respond to insulin stimulation by activating Akt. (A) 75 blastocyst stage embryos were retrieved and immediately incubated for 30 min in HTF/BSA or HTF/BSA supplemented with 1 μM insulin. The embryos were added to Laemmli sample buffer and then subjected to SDS-PAGE and Western blot analysis. The membranes were probed with antibodies specific for p-Akt and actin. This experiment was performed in triplicate with a representative blot presented here. (B) Western blots were quantitated using densitometry and NIH image software, and expression was normalized to actin. The average expression of p-Akt after insulin stimulation was 217% ± 54% compared to untreated control embryos.

tion embryos, blastocysts were cultured in the presence of the PI3K inhibitor, LY-294002. After only 15 min, Akt phosphorylation was inhibited (Fig. 5), again confirming that this pathway responds as expected to environmental cues in preimplantation embryos.

Insulin-stimulated glucose uptake is blocked in the presence of PI3K/Akt inhibitors

Insulin and IGF-1 stimulate glucose uptake in the preimplantation blastocyst specifically via the IGF-1R (Harvey and Kaye, 1991). Since PI3K activity is directly downstream of IGF-1 receptor phosphorylation in most cell types, we hypothesized that PI3K activity may be involved in IGF-1/insulin-stimulated glucose uptake in the blastocyst. To test this hypothesis and to determine the physiologic role of the PI3K/Akt pathway, we analyzed the effect of inhibitors of this pathway on insulin-stimulated glucose uptake in the blastocyst. Exposure of blastocyst stage embryos to the PI3K inhibitors LY-294002 and Wortmannin resulted in the complete inhibition of insulin-stimulated glucose uptake. As shown in Fig. 6, control embryos showed a significant increase in glucose uptake when exposed to insulin. This was in contrast to blastocysts cultured in the presence of either

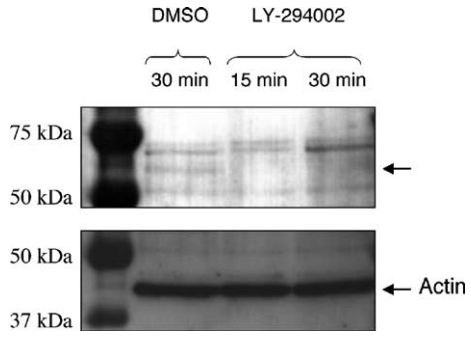

Fig. 5. Akt phosphorylation is inhibited by LY-294002 in the preimplantation blastocyst. Blastocysts were harvested and cultured in either DMSO (vehicle control) or 250 μM LY-294002 for 15 or 30 min and subsequently subjected to SDS-PAGE and Western blot analysis. The membranes were probed with an antibody specific for p-Akt and actin.

LY-294002 or Wortmannin, in which insulin-stimulated glucose uptake was completely inhibited.

Inhibition of the PI3K/Akt pathway results in decreased blastocyst hatching

In mice, the blastocyst hatches from the zona pellucida during the fifth day of development (Nagy et al., 2003). Embryo hatching is required for successful implantation (Hartshorne and Edwards, 1991). To determine whether the PI3K/Akt pathway plays a role in this critical developmental process, we cultured embryos in LY-294002 and determined the percentage of blastocysts hatching at a 24-h time point as compared to controls (Fig. 7). The percentage of blastocysts hatching was 41% ± 3.0% of embryos cultured in DMSO (vehicle control) as compared to 23% ± 3.5% of blastocysts cultured in LY-294002 ($P < 0.02$). Thus,

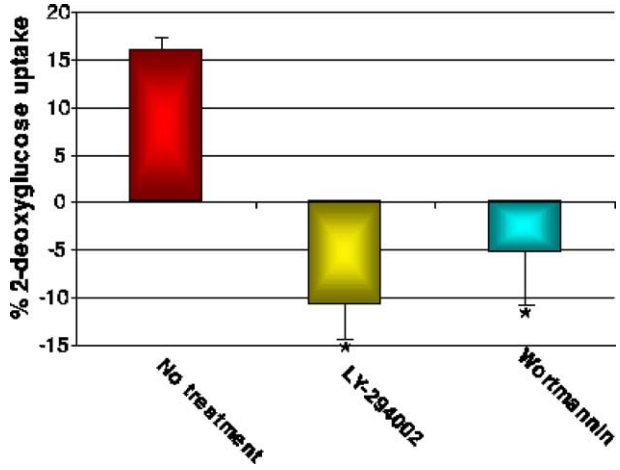

Fig. 6. Insulin-stimulated glucose uptake is inhibited by LY-294002 and Wortmannin. Insulin-stimulated glucose uptake was measured in blastocysts exposed to media alone (no treatment), 250 μM LY-294002 and 100 nM Wortmannin. The percent insulin-stimulated glucose uptake over basal glucose uptake is plotted. Embryos exposed to media alone demonstrated a 15.9% ± 1.2% increase in glucose uptake, while those blastocysts exposed to PI3K/Akt inhibitors demonstrated no increase in glucose uptake in response to insulin with a slight decrease in basal uptake (LY-294002 − 10.7% ± 3.9%; Wortmannin − 5.3% ± 6.6%) (*$P < 0.05$).

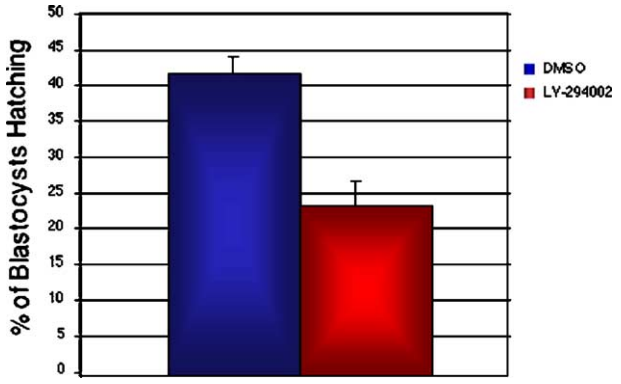

Fig. 7. LY-294002 inhibits blastocyst hatching. Blastocysts were harvested and subsequently cultured in vitro in either DMSO (vehicle control) or 250 μM LY-294002 for 24 h. The percentage of blastocysts hatching after 24 h in culture was 41% ± 3.0% of embryos cultured in DMSO as compared to 23% ± 3.5% of blastocysts cultured in LY-294002 ($P < 0.02$). A minimum of 100 blastocysts were examined per treatment group over the course of three independent experiments.

inhibition of the PI3K/Akt pathway via LY-294002 resulted in decreased blastocyst hatching.

PI3K/Akt pathway is present and functional in trophoblast stem (TS) cells

In an effort to develop an in vitro cell culture system to serve as a model to study the PI3K/Akt pathway in the developing embryo, we characterized the expression and function of PI3K and Akt in trophoblast stem (TS) cells. TS cells are derived from the trophectoderm of mouse blastocysts and possess the capacity to develop into each of the four trophoblast cell lineages (Tanaka et al., 1998). Based on the localization of the PI3K subunits and Akt at the apical surface of the TE in the blastocyst, we believe that the TS cell line would be an ideal in vitro correlate for our studies. As shown in Fig. 8A, the regulatory subunit of PI3K, p85 and Akt is expressed in TS cells. Similar to what was seen in the preimplantation embryo, Akt was constitutively phosphorylated in the TS cells. In addition, exposure of these cells to the PI3K inhibitor, LY-294002, resulted in the inhibition of Akt phosphorylation (Fig. 8B). Taken together, these results recapitulate what was found in vivo and provide a cell culture system to further characterize this pathway during preimplantation embryonic development.

Discussion

The preimplantation stage of development is unique in a number of significant ways. Prior to implantation, the developing embryo is a free floating entity, dependent on autocrine and maternally derived paracrine growth factors for its development. While much is known about signaling pathways that regulate the earliest stages of development in a number of model systems, less is known about the preimplantation period in mammals. In particular, the maternal environment remains ill defined as do the factors that drive these very first stages of development.

Gaining a greater understanding of this stage of development and what growth factors and signaling pathways are utilized are of particular interest in light of the growing field of assisted reproductive technology (ART). Human embryos are routinely manipulated from a one-cell to a blastocyst stage in culture conditions that may or, more likely, may not closely resemble the in vivo environment. In vitro culture can perturb embryo metabolism and gene expression, the long-term consequences of which are unknown. There is mounting evidence to suggest that, while not overt, offspring generated from in vitro fertilization may display developmental and neurological abnormalities (Hansen et al., 2002; Schieve et al., 2002; Stromberg et al., 2002). It is well documented in the field of animal husbandry that offspring generated from embryos exposed to in vitro environments often results in unusually large offspring, referred to as “large offspring syndrome” (Young et al., 1998). In a recent human study, ART was shown to be associated with an increase in the prevalence of the human outgrowth syndrome, Beckwith–Wiedemann

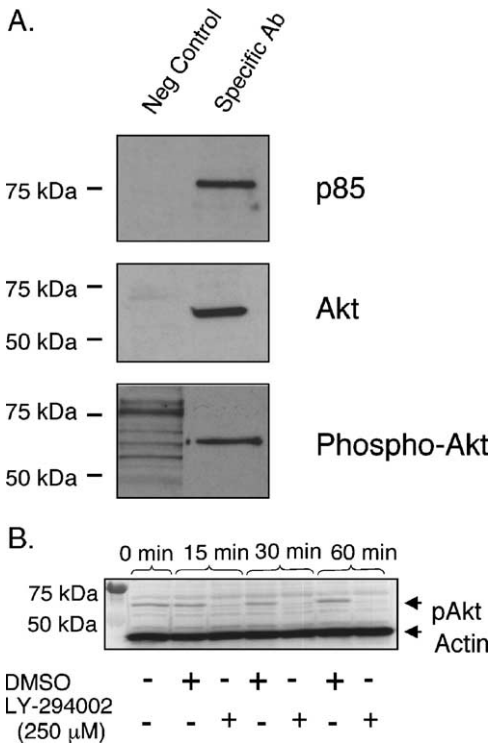

Fig. 8. The PI3K/Akt pathway is present and functional in trophoblast stem (TS) cells. (A) 2×10^6 TS cells were lysed and then subjected to SDS-PAGE and Western blot analysis. 10 μg of protein was loaded per lane. The membranes were probed with preimmune sera or antibodies specific for p85, Akt or p-Akt. (B) TS cells were cultured in either DMSO or 250 μM LY-294002 for 15, 30 or 60 min and subsequently subjected to Western blot analysis. 10 μg of protein was loaded per lane. The membranes were probed with an antibody specific for p-Akt and actin.

syndrome (DeBaun et al., 2003). Finally, adult mice derived from embryos cultured in vitro exhibited significant cognitive deficits as compared to control animals (Ecker et al., 2004). It is for these reasons that it is critical to understand how early development is regulated and how perturbations in this environment can have long-term detrimental consequences.

In this manuscript, we established the presence and functionality of the PI3K/Akt pathway during murine preimplantation embryo development. The PI3K/Akt signaling proteins are expressed at all stages of preimplantation development. Based on immunofluorescent imaging of embryos, these proteins appear to be predominantly localized to the PM, suggesting constant stimulation of this pathway during the preimplantation period. Presumably, maternal- and/or embryo-derived growth factors are responsible for this ongoing activation. At the blastocyst stage of development, we demonstrate that this pathway is functionally active as shown by the increased phosphorylation of Akt in response to insulin stimulation. Additionally, we were able to inhibit this response with the PI3K inhibitor, LY-294002.

Insulin and IGF-1 stimulate glucose uptake in the preimplantation blastocyst via activation of the IGF-1R (Harvey and Kaye, 1991). Previous work from our laboratory has shown that down-regulation of the IGF-1R results in the inhibition of insulin-stimulated glucose uptake (Chi et al., 2000). It was concluded from these studies that the defect resulting in the inhibition of glucose uptake was a direct result of the decreased downstream signaling from the IGF-1R. Data presented here confirm that the IGF-1R is upstream of PI3K/Akt and that insulin-stimulated glucose uptake is PI3K-dependent at the blastocyst stage of development, similar to what is found in adult muscle and adipose tissue. Recently, Gross et al. (2005) have demonstrated another possible physiologic consequence of the PI3K pathway by showing that inhibition of this pathway leads to ES cell and blastocyst apoptosis. These experiments, however, were performed under serum-free conditions, which alone can induce apoptosis. Under physiologic conditions, other growth factors, either present in the embryo's milieu or produced by the embryo itself, may compensate for changes in PI3K activity. These findings suggest that withdrawal of growth factors and decreased signaling via the PI3K/Akt pathway may trigger apoptosis as shown in cell systems such as neurons and lymphocytes (Pierchala et al., 2004; Plas et al., 2002). Further studies are necessary to elucidate the physiologic consequences of PI3K/Akt inhibition and the mechanisms involved.

Importantly, we demonstrated a developmental consequence of the PI3K/Akt pathway by showing that inhibition of this pathway leads to decreased blastocyst hatching which is required for successful implantation of the embryo. Previous studies have identified factors that affect mouse embryo hatching. Huang et al. demonstrated that COX-2-derived prostacyclin increases mouse embryo hatching. A COX-2 inhibitor was shown to inhibit embryo hatching; however, this inhibition was reversed if iloprost, a prostacyclin analogue, was also added to the media (Huang et al., 2004). The mechanism by which inhibition of the PI3K/Akt pathway results in decreased blastocyst hatching remains unclear. However, recent studies have demonstrated that the PI3K/Akt pathway may regulate COX-2 expression at least in certain cell lines (Sheng et al., 2001; Tang et al., 2001; Weaver et al., 2001). Whether or not inhibition of PI3K in the embryo has an affect on COX-2 expression and thus embryo hatching remains to be determined.

The PI3K/Akt pathway is a well-known mediator of growth promoting and cell survival signals. Evidence suggests that PI3K-generated phospholipids regulate Akt activity by directly binding to its PH domain (Franke et al., 1997). A consequence of Akt binding to phospholipids is the translocation of Akt from the cytoplasm to the inner surface of the plasma membrane (Currie et al., 1999). Relocalization of Akt to the PM brings Akt into proximity of regulatory kinases that phosphorylate and activate Akt. Interestingly, at the blastocyst stage, the point at which the first differentiation step has occurred and two cell types, the ICM and TE, are present, PI3K and Akt localize to the PM of the apical trophectoderm. It is this cell layer that directly interacts with the maternal environment and is responsible for transferring external stimuli into the developing embryo.

The localization of the PI3K/Akt signaling molecules to the TE of the blastocyst led us to investigate the presence of this pathway in trophoblast stem (TS) cells, potentially providing us with an in vitro cell line to study this pathway in embryonic development. Protein expression of the regulatory subunit of PI3K, p85 and Akt was demonstrated by Western blot analysis of TS cell lysates. Similar to what was seen during preimplantation embryo development, Akt was constitutively phosphorylated in the TS cells. Moreover, the PI3K inhibitor, LY-294002, abrogated constitutive Akt phosphorylation. Our results are similar to what was shown in a previous study, which characterized the role of the PI3K/Akt pathway in the differentiation of trophoblast cells (Kamei et al., 2002). This group found, as we did, that Akt is constitutively active in TS cells. In addition, they were able to show that there was an increase in Akt phosphorylation when the cells were forced to differentiate. Taken together, these data are the first to demonstrate that the PI3K/Akt pathway is present and functional at this early stage in development.

This manuscript provides a comprehensive analysis of the presence and function of the PI3K/Akt signaling pathway during preimplantation development. The data establish the role of this pathway in IGF-1-receptor-regulated glucose uptake as well as in blastocyst hatching to facilitate implantation. Further analysis is underway to determine how physiologic perturbations in this pathway affect the developing embryo.

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